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Environmental Requirements for Sporocarp Germination in Marsileaceae

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Environmental Requirements for Sporocarp Germination in Marsileaceae

(TITLE)

BY

Kevin J. Kruep

THESIS

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ABSTRACT

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Experiments were conducted on sporocarps of *Marsilea quadrifolia L.* to determine whether temperature had any influence on the length of the reproductive period and initial sperm emergence from microgametophytes. Data were then compared to results of similar studies undertaken with the species *M. vestita* Hooker and Greville to determine whether there was any variation between them. A slight variation was recorded, ranging from 1 to 1.5 hours for initial emergence.

An additional set of experiments was performed with 58 year old sporocarps and approximately two-year-old sporocarps of *M. quadrifolia.* A difference of 2-6.5 hours for initial sperm emergence occurred between the two collections, suggesting that age did have an effect on the onset of emergence.

Sperm population life spans were also monitored, as well as individual sperm life spans, and it was found that a range of 25-30°C was the optimum temperature for sperm survival. Variation in sperm population life spans was also shown between the 58 year old and two-year-old collections, with an average range of 1-1.5 hours. Life spans for individual sperm averaged 45 minutes in all experiments.

An additional test was performed to see if the presence or absence of light had any effect on the time of sperm emergence. The experiment was conducted on sporocarps *of M. quadrifolia* at 25°C. It was determined that illumination was not a factor in the process of sperm emergence. Sporocarps of additional species of *Marsilea* were obtained from the Missouri Botanical Garden and tested for variance in sperm emergence periods. The experiments were performed at 25°C and yielded no viable sperm from the four additional species tested.

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INTRODUCTION

Marsilea is a heterosporous water fern belonging to the family Marsileaceae. It is also referred to as "water clover" because its life cycle is dependent on the presence of water, and the appearance of its leaf arrangement is reminiscent of a four-leafed clover (see Fig. 1). The family also includes the "pillworts" (*Pilularia L.*) and the monotypic *Regnellidium* Lindman, which produces a latex substance through its physiological processes (Johnson, 1986). *Marsilea* includes about 60 species widely distributed in the temperate and warm regions of both hemispheres. Most of the seven United States species are confined to Texas, but *M. quadrifolia* occurs in the midwest to northeastern while *M. vestita* is western in distribution (Lellinger, 1985).

M. quadrifolia, a species introduced to the U.S. from Europe at around 1860 in Connecticut, is commonly found today in the midwestern states of Illinois and Indiana up through the eastern seaboard (Johnson, 1993). It was chosen for this research based on its availability and known presence in this region. Reproductive structures were obtained from two collections of plant material representing two different ages of sporocarps.

Marsilea reproduces by way of a sporocarp, a sclerified seed-like propagule found at the base of the petiole at each node of its creeping

rhizome (Gifford and Foster, 1987). This sporocarp can lie dormant for many years and then "germinate" when conditions are favorable. *Marsilea* can be found growing at the edge of lakes and ponds on the muddy substrate as well as in the water floating on the surface. In both cases, however, its stays firmly rooted in the mud and never becomes a free-floating aquatic plant as do the other heterosporous water ferns in the Salviniales, *Salvinia* and *Azolla.*

The study of the reproductive processes of *Marsilea* is intriguing because we can easily watch the process of fertilization take place in a laboratory setting. This process actually allows one to observe sperm emerging from the microgametophyte and watch them swim about until they find and fertilize the egg in the archegonium of the megagametophyte. Another interesting attribute of *Marsilea* is the reproductive propagule called the sporocarp. This structure contains all of the material neccessary for the complete reproduction of *Marsilea,* compacted into a tiny sphere or obovoid about the size of a fertilizer pellet. This process is very reliable and has been observed each semester for the past 58 years by EIU morphology classes to provide an example of fertilization within the family Marsileaceae. *M. quadrifolia* was chosen as the species to investigate whether sperm emergence periods and sperm population lifespans within *Marsilea* show consistent results compared to other documented species.

The objectives of this study are to 1) investigate the effects of temperature on the reproductive period and lifespan of *Marsilea* sperm; 2) compare the data of *M. quadrifolia* to the data *of M. vestita* and to other populations(i.e. inter- and intraspecific comparisons); and 3) study the influence of light on the time required for sperm emergence and fertilization; 4) obtain and test other species of *Marsilea* to test for any variation in sperm emergence periods; and 5) investigate the effects of freezing on sporocarps in order to explore the pheasability of collecting more material from herbarium specimens.

LITERATURE REVIEW

SPOROPHYTE MORPHOLOGY

The growth habit of *Marsilea* does not greatly resemble that of other ferns even though it is classified as a fern included in Division Filocophyta. In terrestrial species, *Marsilea* forms diffuse or dense colonies that spread along the ground on creeping rhizomes that are anchored to the substrate by a dense, adventitious root system located at the nodes of the rhizome and occasionally in the middle of an internode, depending on the species (Johnson, 1986). Aquatic forms are either floating or emergent, in either case they are rooted in the soil substrate.

The leaves of *M. quadrifolia* are quadrifid, resembling those of a four-leaf clover (Fig. 1) with the young leaf circinate in vernation and the pinnae folded together until late in development (Gifford and Foster, 1987). The leaves are supported by a petiole that ranges from 5 - 16 cm in length and occurs at the node of the creeping rhizome (Johnson, 1986). Leaves differ between the water and land forms in that the water form leaf has a divided lamina that is expanded in the plane of the petiole, while the land form is quadrifid and is expanded at right angles to the petiole. Allsopp (1963) has related these differences primarily to nutrition. The leaves show great plasticity of form in

response to a wide range of physical and chemical stimuli, such as far-red light (Gaudet, 1963), CO_2 concentration (Bristow and Looi, 1968), protein synthesis inhibitors (White, 1966), gibberelins (Allsop, 1959), and abscisic acid (Liu, 1984). Floating leaves in *Marsilea* vary from simple to four-parted, but are uniformly flattened, and have stomata only on the adaxial surface (White, 1971), and are small and delicate compared to other leaf forms. The floating leaves have long lax petioles and leaflets that lie flat on the water's surface and are splayed out in a circular configuration. These modifications help the leaf maximize its ability to float on the water while at the same time reducing the damage caused by wave action (Johnson, 1986).

The terrestrial leaves of *Marsilea* are reduced in size and are hairier, have abaxial stomata, and have stiffer petioles. Unlike the aquatic forms, the terrestrial form's leaves and petioles do not display or possess any color pigmentation of dark red blotches distributed about the surface, as in the aquatic forms.

The rhizome in all species of Marsileaceae is terete and varies from parenchymatous and glabrous in water to sclerified and hairy in the land species (Johnson, 1986). Its growth habit consists of a long, creeping structure that spreads itself out across the ground with node/internode construction. The petioles arise at the nodes, using the node for support and as a starting point for growth. Growth of the rhizome is generated by a uniform unit of growth with each unit being

formed from the same meristem, rather than by separate ones (White, 1984).

The sporocarp stalk (also referred to as a peduncle) is attached to the petiole at, or considerably above, its base. The sporocarps begin to develop later than the attached leaf, and often do not mature until after the leaf has withered or has broken away (Johnson, 1986). When the sporocarp is immature it is leathery in texture and is green and photosynthetic. As it matures, it turns a brownish black and becomes more solid. Mature sporocarps are bilaterally symmetrical and have grown to 9 mm long, 6 mm wide, and 2.5 mm thick (Johnson,1986). The hard wall, consisting of a cutinized epidermis, two layers of columnar sclerids, and a layer of I-shaped cells ensures its viability over long periods (Allsop, 1952). These specialized structures are thought to be laminar in evolutionary origin. Whether the sporocarp represents a simple, folded basal pinna, or through evolution, arose as an entire pinnate leaf, has yet to be determined (Foster and Gifford, 1987).

The viability of some sporocarps have been reported up to 100 years, as is the case with sporocarps of *M. oligospora* Goodding collected from plant material housed at the Gray Herbarium (GH) (Johnson, 1986). The maximum period a sporocarp can remain viable has yet to be determined. Many germination attempts have been made with sporocarps extracted from even older collections, but the microwaves

now sometimes used for specimen sterilization are thought to be the reason that the sporocarps are no longer viable. For those specimens that are merely frozen, as is also often the practice, the cold temperatures do not seem to have any effect on the viability of the sporocarps. Since there is no way of knowing which sterilization technique was used in the past, herbarium specimens can sometimes be used successfully as a source for additional material.

PHYSIOLOGY

The reproductive processes of *Marsilea* can be completed in under six days. The sporocarp can withstand dessication because of its hard outer surface and it can maintain itself in the environment in an inactive (dormant) state for long periods of time before any emergence occurs. The process of fertilization begins with the physical scarring or breaking of the hard outer wall and the subsequent hydration of internal components of the sporocarp (i.e., the sorophore, microspores, and megaspores). This occurs when the protective coat of the sporocarp breaks down from prolonged exposure to water or an abrasive surface. Upon hydration of the internal components, a gelatinous mass of pectin (the sorophore) emerges from the sporocarp (see Fig. 2, C). The sori, which hang from the lower surface of the sorophore on pinnae contain two types of spores; the microspores and

megaspores. The sporocarp contains, on average, 112 megaspores (no data is available for the number of microspores). The microspore and megaspore are the male and female reproductive components (respectively) of the sporocarp. The haploid microspores develop into the endosporic microgametophyte and produce the sperm that will fertilize the single egg found in the archegonium of the megagametophyte (Fig. 2, F). The time it takes for the sperm to emerge from the microgametophyte depends on several environmental stimuli, most importantly, temperature. The time from initial rupture of the microspore wall to the release of sperm from the single antheridium averages about 30 seconds to 1 minute.

Upon emergence from the antheridium, the sperm will begin to move in the water in a spinning motion and in a clockwise fashion, propelled by numerous flagella. Flagella, numbering between 90-100 per individual sperm, propel the sperm allowing it to reach its destination--the sperm lake adjacent to the archegonium in the anteriorly-based gelatinous mass found encasing the megagametophyte. When the sperm reach the interior of the sperm lake they move toward the archegonium and burrow through its surface to the egg and fertilize it (Rice and Laetsch, 1967). It is unknown exactly how long the sperm require to fertilize the egg, but the first macroscopic signs of sporophyte growth can be seen at six days.

The *M. vestita* Hooker and Greville studies relating to the ultrastructure of sperm indicate that there are two main structural components of an individual sperm. They consist of a posterior cytoplasmic vesicle and an anterior nucleus- and flagella bearing coil containing 9-11 gyres (Rice and Laetsch, 1967). Starch grains and mitochondria are found in the vesicle, which is believed to be the source of energy for the sperm. The main structural components of the sperm coil are a continuous mitochondrial band, an elongate nucleus, and a series of microtubules that separate the basal bodies from the nucleus and mitochondrion (Rice and Laetsch, 1967).

An individual sperm's life is relatively brief, lasting about 45 minutes to an hour; sperm populations last from 3.5 hours to 4.5 hours. Senescence is first evident by a gradual loss of sperm motility. The direction of rotation either reverses itself or maintains its previous clockwise motion. Final senescence is evident when the sperm stops any directed movement and slowly spins on its axis. Shortly after ceasing its rotational movement, the sperm will release its coil by unraveling it and then detaching it from the cytoplasmic vesicle. A gradual breakdown of the coil occurs after being released from the vesicle.

The water fern *Marsilea* can occur as an aquatic form or as a terrestrial form depending on conditions in the environment, or on cultural conditions in the laboratory. Previous investigators have

demonstrated that the growth of land forms in *Marsilea* can be altered by many factors. Allsopp (1955) concluded that sugar concentration in the culture medium was a major determinant of growth form in the sporophyte of all species of *Marsilea*. In a medium containing a low concentration of glucose, the plant grew as a typical aquatic form, whereas increasing the osmotic potential of the medium by increasing the level of glucose, or by adding mannitol, led to the development of the terrestrial form. In a later work (Allsopp, 1963) the formation of the aquatic form was induced by the addition of gibberellic acid (GA) to a high sugar medium in which a terrestrial form was normally produced.

The (relative) intensity of light on the growth of *Marsilea* sporophytes (after fertilization) has a profound effect on whether it will become a land or water form. *M. vestita* plants placed in darkness or under far-red light became etiolated (Gaudet, 1963). These plants had certain characteristics that are seen in the terrestrial forms, such as a large number of stomatal initials on both surfaces of the leaf. The lamina and petioles were thin and elongated, but differed from both the land and the water form in that the leaf lamina did not expand and the rhizome was ageotrophic. Unless the plant was given red light it never developed into either the terrestrial or aquatic form (Gaudet, 1963). This study suggests an additional fact relating to the influence of light on the growth of *Marsilea;* plants that grow in the shade are likely

to become sterile, whereas plants that grow in direct sunlight have a better chance of reproducing under normal conditions.

ANATOMY

The outer cortex of the rhizome of *Marsilea* is arenchymatous, with 20-30 longitudunal air channels separated by septa 4-5 cells long and 1-2 cells wide (Johnson, 1986). Air channels are unknown among most ferns but are common features of underwater organs of vascular hydrophytes, so much so that Ogden (1974) chose them as the character with which to define aquatic plants. These air channels have diaphragms located throughout the rhizome to help resist the collapse of the channel and reduce the flooding of broken plant parts and also provide for transport of nutrients across the cortex (Tomlinson, 1982).

The vascular cylinder of *Marsilea* is an amphiphloic siphonostele with both an internal and external endodermis and simple leaf gaps; however, in condensed lateral shoots the short internodes may give rise to a dictyostele. Xylem maturation is reported to be exarch (Smith, 1938) or mesarch (Bierhorst, 1971) depending on the diameter of the rhizome. The amount of xylem in the rhizome of *Marsilea* varies within the genus; as in other aquatic groups, such as the Alismatidae (Tomlinson, 1982), the more aquatic members have less vascular tissue and less lignification of tracheary elements than the

more terrestrial members. The tip of the rhizome houses a tetrahedral apical cell that produces the segments of the shoot. The inner portion of the cortex has compact tissue while the outer portion is lacunate with large air spaces around radiating rows of parenchyma (Gifford and Foster, 1987).

Root growth within *Marsilea* is abundant, with roots commonly reaching 50 cm in length. Lateral roots can be absent but, if present, are relatively short; some species have plumose roots which provide an increased surface for nutrient uptake. The outer cortex of the root is aerenchymatous while the internal cortex is parenchymatous and is usually dark due to the dark red pigment that is present. The vascular cylinder of the root of *Marsilea* shows a small diarch protostele. All New World species have metaxylem elements with differentiated end walls, varying from only oblique and scalariform in *M. deflexa* A. Braun and *M. crotophora* D.M. Johnson to simple perforation plates in most other species (Johnson, 1986). *M. macropoda* Englemann ex A. Braun and *M. drummondii* A. Braun have curious half-vessels, with a simple perforation plate present at one end and completely lacking at the other.

The leaves of *Marsilea* show remarkable plasticity of form. The type of leaf formed is related to the size and nutritional status of the shoot apex (Allsopp, 1963), and roughly parallels the heteroblastic sequence of leaves produced by the young plant. Like the rhizome, the

petioles have cortical air channels seperated by thin septa or diaphragms. The petiole is normally terete, but in certain species has a bulbous swelling at its apex close to the attachment of the leaflets that possibly assists in flotation (Senn, 1909).

In cross section, the leaflets have a uniseriate epidermis, a palisade layer, a spongy layer with vascular bundles, a thin region of aerenchyma, and a lower epidermis that lacks stomata. Hildebrand (1870) found that the stomata on the upper epidermis of floating leaves were denser than on the upper epidermis of terrestrial leaves in *M. quadrifolia* and *M. strigosa* Wildenow. Compared to the floating leaves, the land leaves of *Marsilea* are smaller and hairier, have stiffer petioles, have abaxial stomata, may have crenate terminal margins of the leaflets, and have proportionally smaller air channels in the petioles (Johnson, 1986). The land leaves have fully functional pulvini, which manipulate the angle of the leaves toward sunlight during the day and cause the leaves to close at night. The vascular strand of the petiole is V-shaped and is bounded by an endodermis. Within the strand, the xylem is also V-shaped. Selection in the evolutionary history of the Marsileaceae may thus be seen as having favored a leaf of a form adaptable to functioning as a floating leaf; because of the developmental constraints, this design influences the form of the land leaf as well (Johnson, 1986).

Inside the wall layers of the sporocarp is an inverted V-shaped trace surrounded by collenchyma (Johnson, 1986). Lateral veins extend from this trace its entire length and supply the branched or unbranched receptacles of the sori. The sori are also attached to a mass of gelatinous tissue, the sorophore, just below the median trace, and are torn from their vascular moorings when the sorophore becomes hydrated, thereby expanding and pushing the sori out of the sporocarp (Johnson, 1986).

PREVIOUS GERMINATION STUDIES

Several previous studies on sporocarp germination have been performed. The most notable and rigorous experiments were completed by Harbert Rice and W.M. Laetsch (1967) with sporocarps of the species *M. vestita,* the particular species common on the campus of the University of California at Berkeley where they resided. Their experiments revealed that spermatid development was temperature dependent, and that 22-25°Celsius was the best range for maximum sperm emergence.

The *M. vestita* experiments were performed at eight temperatures at five degree intervals starting at 5° Celsius up through 40° C. The time it took for sperm to emerge from the microgametophyte was recorded at each interval and then graphed to

show variation across the range of temperatures. The data showed that at lower temperatures the time it took sperm to emerge from the microgametophyte was longer than at the higher temperatures. Sperm remained viable up to 40° C, but sperm discharge was sporadic at 35° and 40° C. The experiment also revealed that there was no sperm activity at the lowest temperature of 5° C.

Sperm life spans were recorded using motility and O_2 uptake as criteria and it was found that sperm populations were active the longest (3.5 hours) at 25° C for (Rice and Laetsch, 1967). Individual sperm life spans were also monitored; 45 minutes was the average life span of individual sperm.

A separate study on the viability of megaspores, in the absence of microspores, was conducted by Paul Mahlberg and Margaret Baldwin (1975) at Indiana University at Bloomington. Megaspores of *Marsilea vestita, Pilularia americana* A. Braun, and *Regnellidium diphyllum* L. were tested, and there was a 50-75% viability rate when grown in the presence or absence of sporocarp contents. With *Marsilea,* sporophytes formed on 58% of the viable megagametophytes when in the presence of microgametophytes and sperm. Their study also showed that archegonium receptivity to fertilization progressively decreased when the archegonia were not fertilized within approximately 12-24 hours, and that no fertilization occurred after 24 hours.

MATERIALS AND METHODS

Sporocarps of *M. quadrifolia* were obtained from two collections, Eastern Illinois University-Campus Pond and Kickapoo State Park lake. Herbarium specimens from each collection were deposited in the EIU herbarium. The sporocarps from the EIU campus pond site were stored at room temperature in an unsealed mason jar. The sporocarps of the Kickapoo collection were retrieved from the muddy substrate at the collection site. Both sets of sporocarps were rinsed repeatedly with sterilized, distilled water before any treatment began.

The sporocarps were sterilized in a 1% Hypochlorite solution for five minutes and rinsed several times with sterilized, distilled water (Rice and Laetsch, 1967). The sporocarps were air dried on filter paper and the sporocarp coat nicked with a sterile razorblade to accelerate the hydration of the sporocarp contents (Rice and Laetsch, 1967). Four nicked sporocarps each were placed in four 250 ml beakers that contained 200 ml of distilled, sterilized water, and the beakers were placed in a Sherer Model CEL 25-7 controlled environment chamber. The growth chamber temperature was set at five degree intervals (10°, 15°, 20°, 25°, 30°, 35°, 40°C) for each of the seven experiments and the chamber was monitored frequently with a centigrade thermometer in order to maintain growth chamber temperature. The sporocarps were observed until the sorophore was fully extended (approximately 5cm in

length) in the water and the microgametophytes and megagametophytes were released from the sori. Frequent examinations of the water were made for any signs of spermatid presence by removing megagametophytes and microgametophytes from each of the four beakers with an eyedropper and placing them on a glass slide for microscopic observation. This process was performed every half hour until the presence of sperm was evident. After sperm emergence, the micro- and megagametophytes were checked every 15 minutes to monitor the activity of the sperm and to record the amount of time sperm and sperm populations remained viable. After emergence ceased, the sperm were viewed until most of the sperm coils were released from the cytoplasmic vesicle and all motion stopped. The beakers were then set beneath a twin flourescent lamp light source and checked daily for possible sporophyte growth from the archegonium of the megagametophyte.

This process was repeated for each of the seven experiments. All data were then tabulated and graphed with Harvard Graphics computer spreadsheet software.

An additional set of experiments was conducted in the absence of light to see if darkness had any effect on the reproductive cycle of *M. quadrifolia*. All processes were identical to those above, with 25°C the optimum temperature for maximum sperm emergence.

Another set of identical experiments was performed with the two-year-old Kickapoo State Park sporocarps, in order to compare the results with the 58 year old EIU collection.

Sporocarps of *M. quadrifolia* were also used to investigate whether the exposure of extreme cold had any effect on sporocarp germination periods. The sporocarps were wrapped in gauze with an outer envelope of alluminum foil and placed in a freezer and left for two weeks. The sporocarps were removed and tested identically to previous experiments at 25° C to determine if the cold temperature affected the viability of the internal components.

RESULTS AND DISCUSSION

The results of the experiments performed on the *M. quadrifolia* sporocarps obtained from the EIU Campus Pond showed some variation when compared to the *M. vestita* sporocarps used in the Rice-Laetsch experiments (1967)(Fig. 3). The temperature neccessary for maximum sperm production of the two species closely paralleled each other with anywhere from 1 to 1.5 hours in difference between temperatures of 15° and 30°C. For *M. quadrifolia* there was no sperm emergence at 10°, 35°, and 40° C.

At 15° C, initial sperm emergence occurred at 25 hours (Fig. 3). There were very few sperm present throughout the experiment, probably due to the cool temperature. The life span of the sperm population was approximately 2.5 hours (Fig. 4). At 20° C, sperm emergence occurred within 11.5 hours (Fig.3) showing a 54% faster emergence period for a 5 degree increase in temperature. This demonstrated that the warmer temperatures decreased the amount of time necessary for sperm to emerge. The life span for the sperm population also increased by 15 minutes, suggesting that increased temperatures improve sperm population life spans. At 25° C sperm emergence occurred at 8.5 hours indicating a 26% faster emergence rate for another 5 degree increase in temperature. The life span of the population increased another 15 minutes, to 3 hours. In addition, more

sperm were observed at this temperature than at any other temperature, reinforcing the conclusion that 25° C is the optimum temperature for sperm emergence. At 30° C, the initial emergence of sperm occurred at 6 hours with sperm populations remaining active for 3 hours. For 35 and 40° C there were no sperm activities.

The comparison between the Kickapoo and EIU sporocarps showed differences in initial sperm emergence (Fig. 5). This difference is most likely due to the age difference of the collections. The EIU collection was aged at 58 years old (collected May, 1939) whereas the Kickapoo sporocarps were produced approximately two years ago (collected November, 1996). This suggests that the sporocarps of older collections are still viable, but that they are slower to release sperm, probably indicating that their viability decreases gradually over time. At 10°C, there was no evidence of sperm activity. The largest variation occurred at 15°C with a 6.5 hour difference between the two collections. The sperm lifespans of both collections also showed a variation of one hour, with the Kickapoo sperm population surviving for 3.5 hours compared to the EIU sperm for population (2.5 hours). At 20° and 25° C, there was an average of two hours difference between the collections. The sperm life spans also paralleled each other for these two temperatures with approximately 2 hours difference between the two collections. At 30°C, the Kickapoo population emerged at 5 hours and the EIU sperm at 6 hours, still indicating a variation in the sperm

emergence period. The largest variance in sperm life spans between the two collections occurred at 30°C, with the Kickapoo samples surviving for 4.5 hours and the EIU samples for 3 hours. The survival of sperm for this length of time is attributed to the temperature at which this particular experiment was conducted (30° C), which is near the optimum temperature range. No sperm activity occurred at 35°C and 40°C in either collection.

The absence of light had no effect on the time it took for sperm to emerge from the antheridia for both collections tested, although light does play a role in the development of morphological characteristics of both terrestrial and aquatic forms of the sporophyte of *Marsilea* (Laetsch and Briggs, 1960).

In conclusion, temperature does indeed play a critical role in the time it takes for sperm to emerge from the microgametophyte in *M quadrifolia*. The optimum temperature range of 25°-30°C seems to be the best for the fertilization process. Longer incubation temperatures may indeed slow the process of sperm emergence to the point that the sperm are unable to fertilize the egg before the viability of the megagametophyte ceases at 12 -24 hours, or even keep the sperm from emerging at all. This is a possible explanation for the absence of sperm activity at 10°C. At temperatures above 35°C, the extreme heat may be the reason for the absence of sperm. It is possible that these sperm populations rely on environmental stimuli to initiate the emergence

process and that temperatures above 35°C keep the sperm from emerging from the microgametophyte by "overheating" or killing the sperm within the microgametophyte before they emerge. This would explain why no sperm emergence occured with the 35°C and 40°C experiments. It doesn't, however, explain why Rice and Laetsch (1967) had limited success at these temperatures with their *M. vestita* sporocarps. The conflicting results at 35°C and 40°C between these two sets of experiments could be a difference among these two species. The extremes of hot and cold temperatures experienced by the micro- and megagametophytes in these experiments probably helps to dictate the distribution of each species in nature depending on its environmental requirements. *M. vestita* survives better in the warm, temperate climates of the west and southwest U.S., whereas *M. quadrifolia* has a more narrow range of temperature tolerances and succeeds better in the colder midwest and eastern climates of the U.S.

Additional experiments performed to test sperm viability with sporocarps in excess of 100 years of age did not provide any useful data to compare with the results of the *M. vestita* and *M. quadrifolia* experiments. Sporocarps of *M. botryocarpa* F. Ballard,*M. macropoda* *M. polycarpa* Hooker and Greville, and *M. naschii* Underwood and Britton were obtained from the herbarium at the Missouri Botanical Garden and tested at 25°C, but no sperm emerged in any of the four experiments. Herbarium specimen fumigation techniques, such as

microwave and gas exposure treatments were thought to be the probable reason sperm did not emerge from the microgametophytes. Other workers have successfully germinated sporocarps gathered from herbarium sheets, using collections up to 100 years old (Johnson, 1986). The exposure of the sporocarps to these treatments probably killed the internal components.

The exposure of sporocarps to two weeks of freezing temperature (0°C) did not affect germination periods. The sperm emergence and sperm population lifespans of the EIU collection, being identical to the data derived from *M. quadrifolia* experiments at 25° C.

More experiments should be performed with other species of *Marsilea,* in order to compare the data and determine whether the age of the sporocarps in these different species shows any variation in the viability of their sperm. As for sperm emergence, more research should be done in the area of physiological tolerances of *Marsilea* sperm to determine the factors that cause sperm not to emerge at the temperature extremes. This information would help us to better understand the unusual physiological processes that affects fertilization and reproduction in this "un-fernlike" family of ferns.

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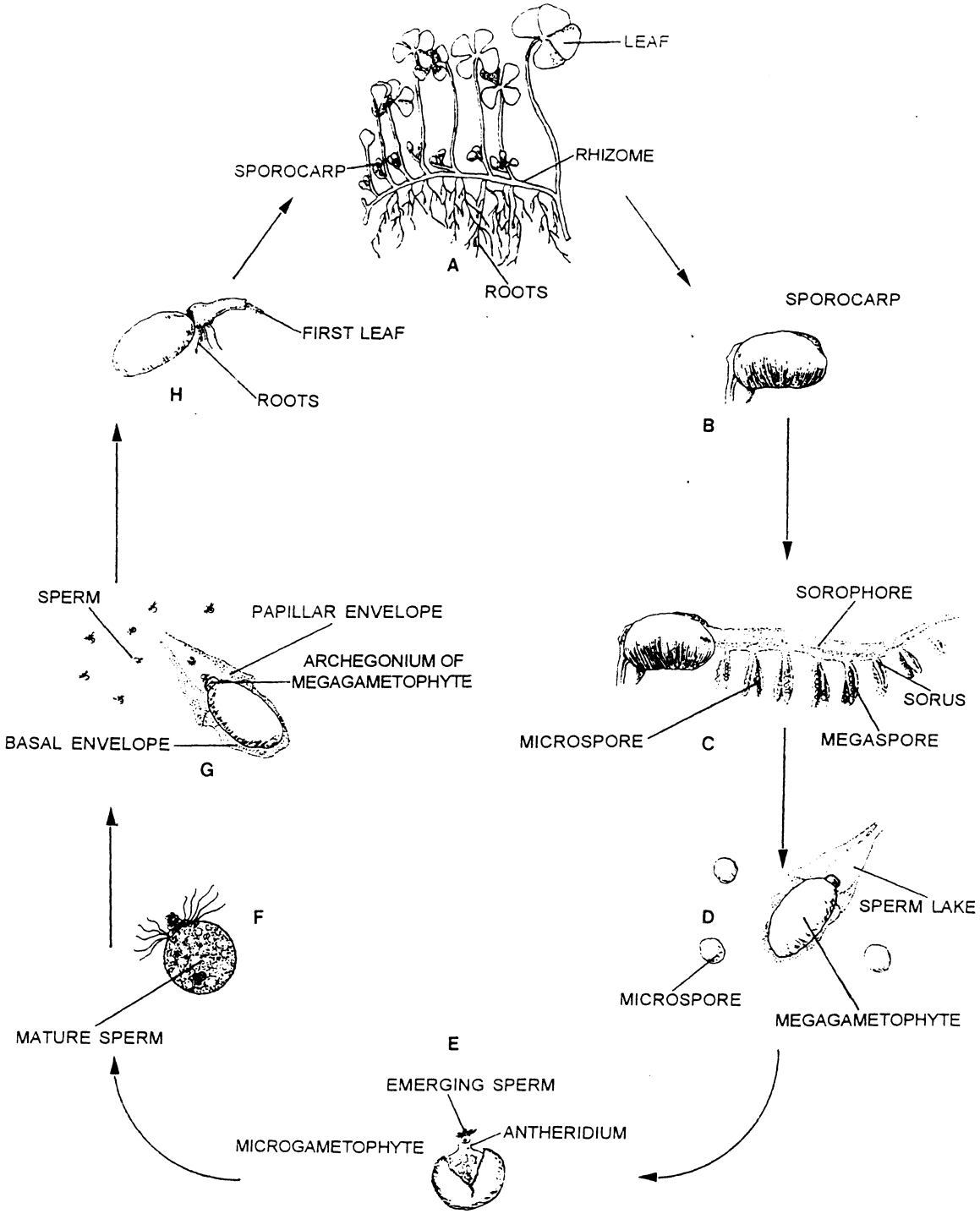
Fig. 1. Marsilea quadrifolia L.



LIFE CYCLE

Marsilea quadrifolia L.

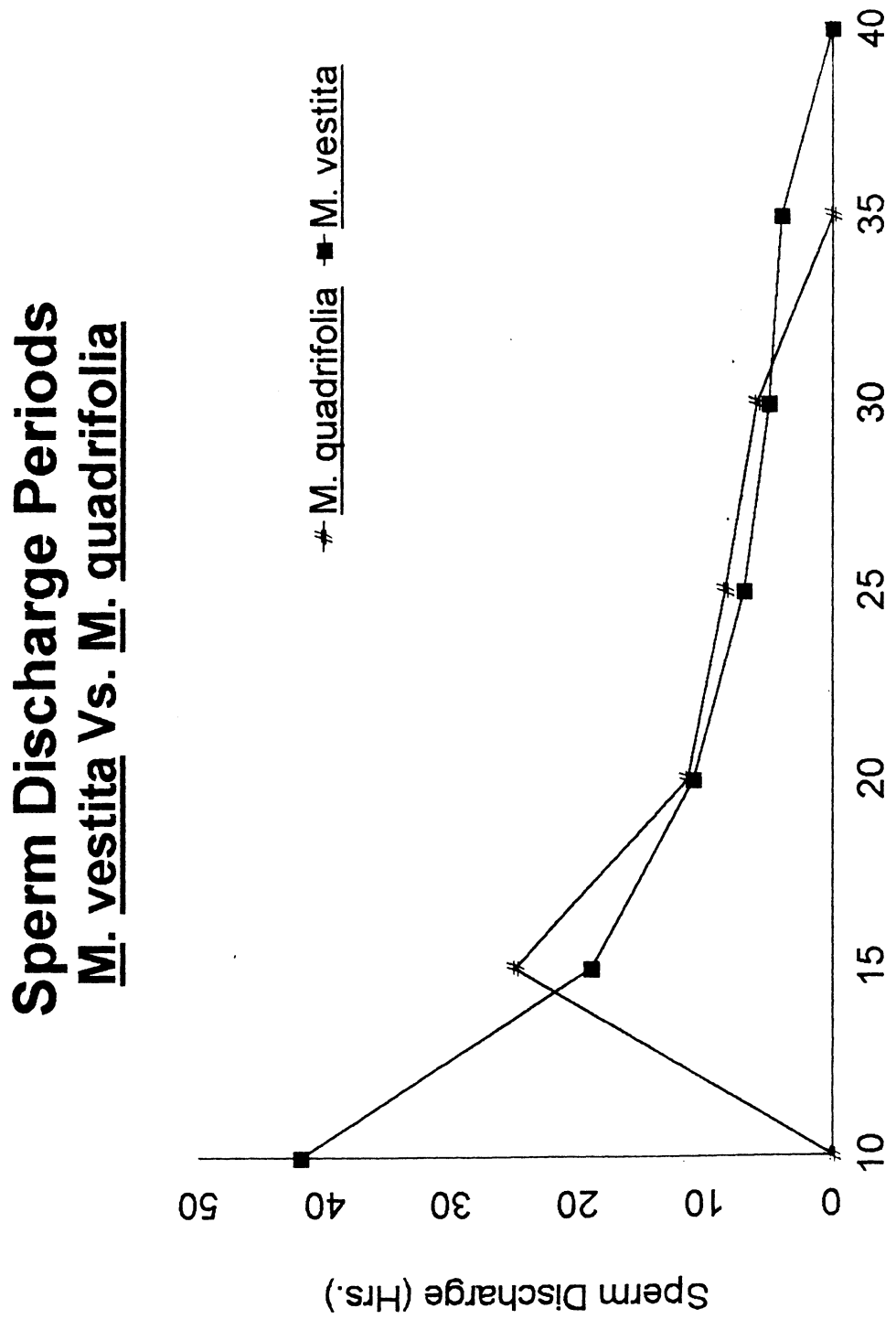

Sperm Discharge Periods
M. vestita Vs. M. quadrifolia
M. quadrifolia
M. vestita
Sperm Discharge (Hrs.)
0
10
20
30
40
50
10
15
20
25
30
35
40
Temperature (C)

Fig. 3

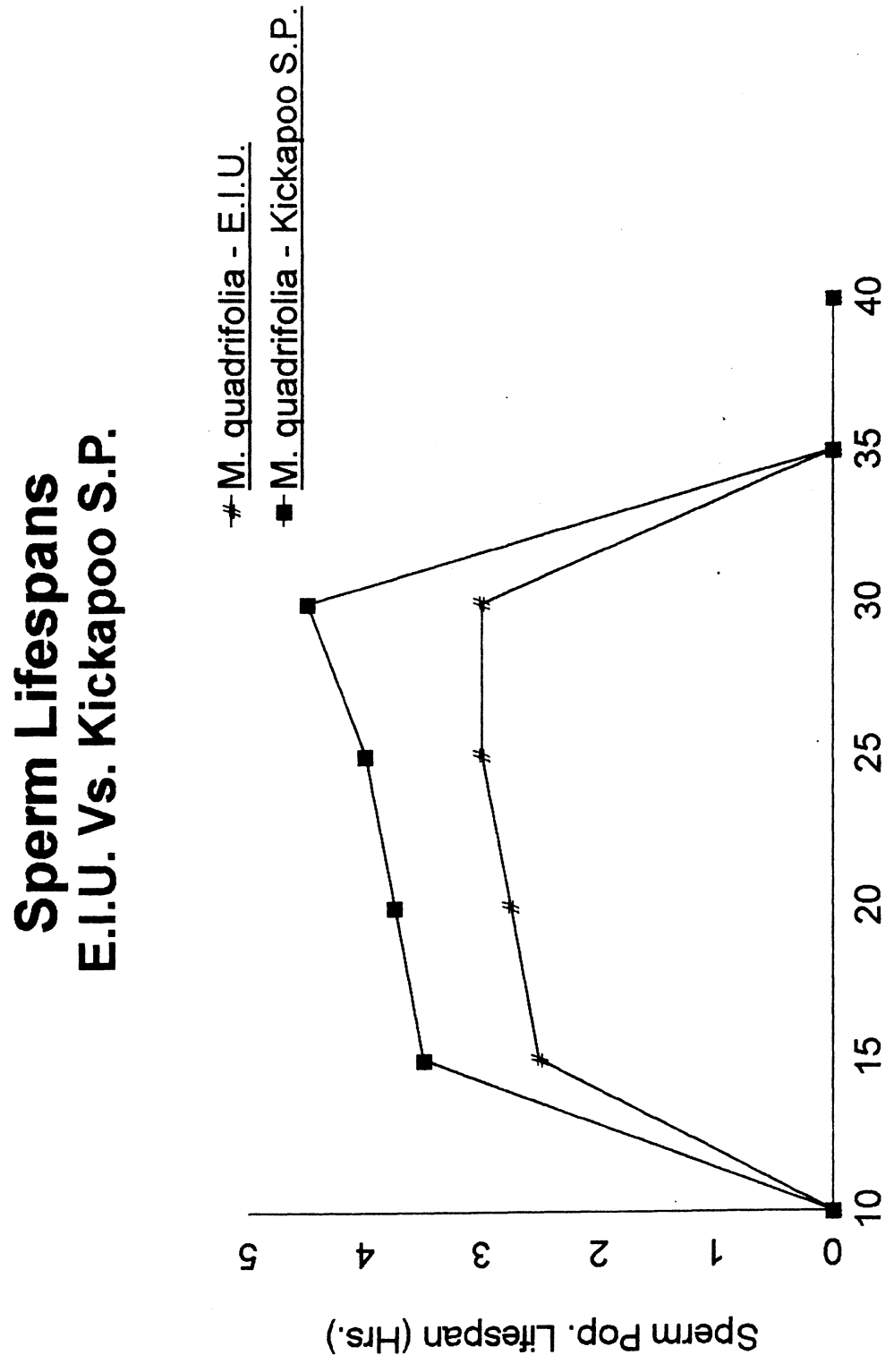

Sperm Lifespans
E.I.U. Vs. Kickapoo S.P.
M. quadrifolia - E.I.U.
M. quadrifolia - Kickapoo S.P.
Sperm Pop. Lifespan (Hrs.)
0
1
2
3
4
5
10
15
20
25
30
35
40
Temperature (C)

Fig. 4

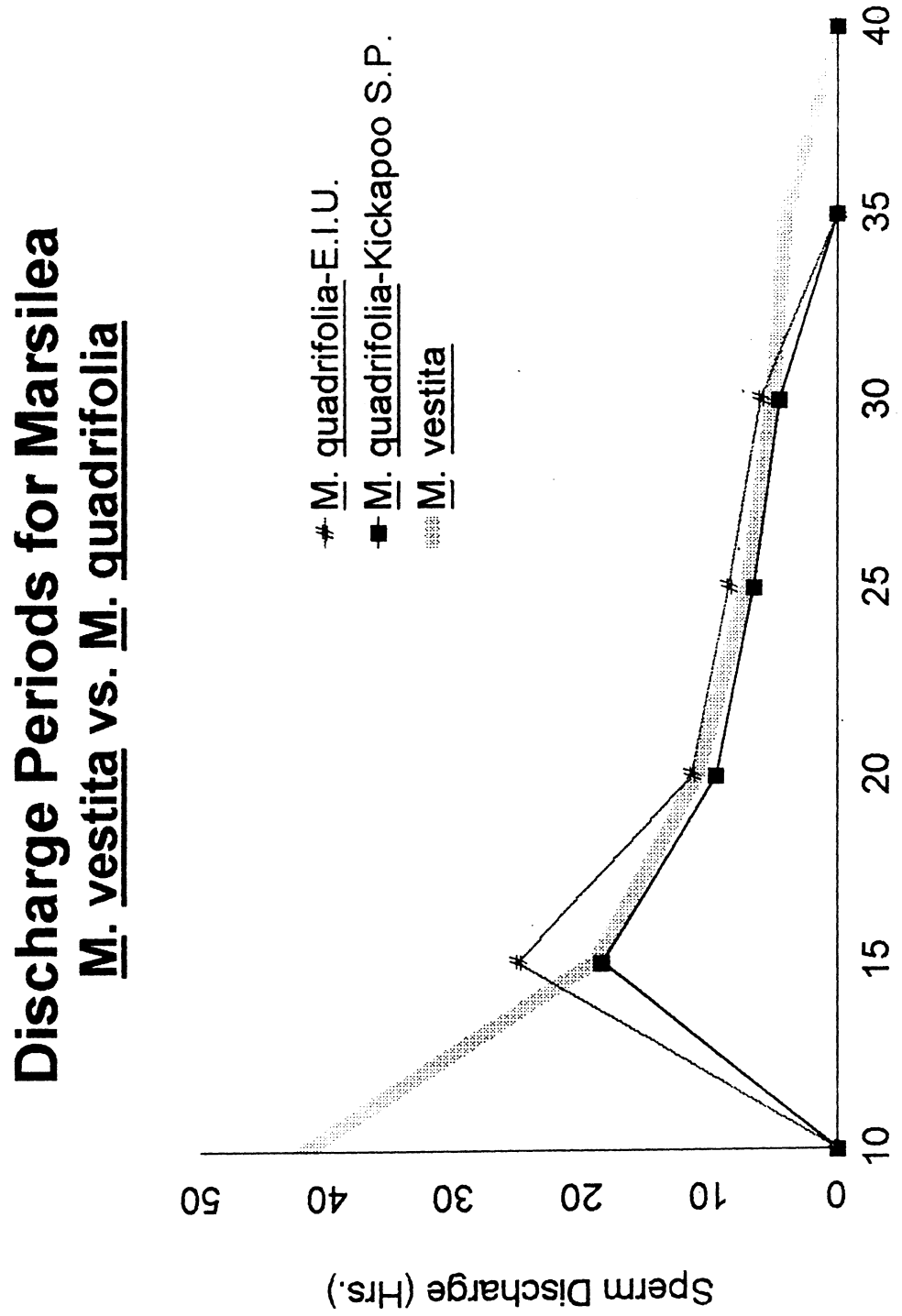

Discharge Periods for Marsilea
M. vestita vs. M. quadrifolia
M. quadrifolia-E.I.U.
M. quadrifolia-Kickapoo S.P.
M. vestita
Sperm Discharge (Hrs.)
0
10
20
30
40
50
10
15
20
25
30
35
40
Temperature (C)

Fig. 5

FIGURE LEGENDS

Fig. 1. Growth habit of *Marsilea quadrifolia* (specimen from the H.F. Thut Greenhouse, Eastern Illinois University).

Fig. 2. Lifecycle of *Marsilea*. A. Vegetative morphology of sporophyte. B. Sporocarp. C. Fully extended sorophore showing sori, microspores, and megaspores. D. Megagametophyte encased in gelatinous envelope showing sperm lake and microspores. E. Microgametophyte exhibiting antheridium and emerging sperm. F. Mature sperm. G. Sperm congregating in sperm lake and fertilizing egg of megagametophyte. H. Megagametophyte showing sporophyte growth with emergence of first leaf and roots. [A,B,C, and H sketches adapted from Foster and Gifford *Morphology and Evolution of Vascular Plants*. W.H. Freeman and Co., New York. 1987.]

Fig. 3. Comparison of sperm discharge time periods between *M. vestita* and *M. quadrifolia*.

Fig. 4. Comparison of sperm population lifespans of EIU and Kickapoo State Park sporocarp collections.

Fig. 5. Comparison of sperm discharge time periods between EIU and Kickapoo State Park collections of *M. quadrifolia* with discharge time periods of *M. vestita*.

APPENDIX A

Species	Collector	Date Col.	Collector #	Site	Herbarium
M. botryocarpa	J.A. Hunn	1973	36	Tsavo Nat'l Park, Kenya	Mo. Bot. Gardens
M. macropoda	Steven Hill	1991	22428	Calhoun County, TX	Mo. Bot. Gardens
M. nashii	D.S. Correl	1978	49945	Great Inagua, West Indies	Mo. Bot. Gardens
M. polycarpa	A. Alston	1939	1867	Bejuco	Mo. Bot. Gardens
M. quadrifolia-E.I.U	Kohlbecker	1938	s.n.	E.I.U. Campus Pond	EIU
M. quadrifolia Kickapoo S.P.	J.E. Ebinger	1996	27123	Kickapoo S.P Vermilion County, IL	EIU